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The proteins of the *Hepatitis C virus*: Their features and interactions with intracellular protein phosphorylation

Brief Review

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Summary. Chronic infection with *Hepatitis C virus* (HCV) often results in cirrhosis and enhances the probability of developing hepatocellular carcinoma (HCC). The underlying mechanisms that lead to malignant transformation of infected cells, however, remain unclear. Observations made with isolated HCV antigens and/or with HCV subgenomic replicon systems demonstrated that the products encoded in the HCV genome interfere with and disturb intracellular signal transduction, often by phosphorylation of cellular proteins. Moreover, some of the HCV-encoded proteins seem to serve as substrates for host cell protein kinases. These phosphorylations affect the biological functions of the antigens. In many cases it could be demonstrated that only short stretches of the linear sequence of the viral or cellular proteins are involved and play a crucial role for these phosphorylation events. The identification of these small polypeptide elements and the subsequent development of strategies to inhibit protein–protein interactions involving them may be the first step towards reducing the chronicity and/or of the carcinogenicity of the virus. This review summarizes current knowledge of intracellular phosphorylation processes that are affected by HCV.

Genome structure of HCV and polyprotein processing

HCV was identified as the causative agent of non-A, non-B Hepatitis in 1989 [19, 20]. Approximately 170 million people are infected with this virus worldwide. At least 85% of the infected patients develop a chronic hepatitis. The major complication of chronic HCV infection is cirrhosis of the liver, which occurs in 20

to 30% of cases. In addition to this, HCV has been shown to be causatively linked to hepatocellular carcinoma as well as some nonhepatic diseases like arthritis, glomerulonephritis, and other autoimmune diseases [35, 68, 83].

HCV is a small enveloped virus, with a linear, positive-sense single-stranded RNA genome, approximately 9.6 kb of size, encoding a single open reading frame (ORF). The HCV ORF is translated into a single polyprotein of approximately 3100 amino acids [64, 84]. Hepatitis C virus closely resembles other members of the family of *Flaviviridae* in terms of genome sequence and organization and mode of replication, as well as in the structure and processing of the HCV polyprotein [71].

The family *Flaviviridae* contains two well-known genera *Flavivirus* and *Pestivirus*. HCV has been classified in a separate genus within this family with the name *Hepacivirus* [55]. The great diversity of the nucleotide sequences within the genus has led to further classification into six major genotypes, numbered 1–6, and numerous subtypes [15]. The genotypes differ in terms their geographic distribution, response to therapy and severity of the disease they cause [32, 72].

The ORF of HCV and other members of the family *Flaviviridae* is flanked by 5′- and 3′-terminally located untranslated regions (5′UTR and 3′UTR respectively). The 5′UTR of HCV is approximately 340 bases long and shows features of an internal ribosomal entry site (IRES) that mediates binding of the RNA to the ribosome [82, 114]. The function of 3′UTR remains to be established. There are some indications that it may be important for the initiation of minus strand synthesis (necessary for the replication of the plus strand) [43] or that the adenosine- and/or uridine-rich stretches of the 3′UTR are involved in the regulation of the NTPase activity of the NS3 associated NTPase/helicase [8]. In the course of HCV infection the viral polyprotein is co- and post-translationally cleaved by both virus-encoded and host cellular proteases (signalases), releasing at least 10 known structural and nonstructural (NS) proteins. The proteins are arranged in the following order: NH_2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B NS5A-NS5B-COOH [17, 74, 86, 98]. The initial cleavages, which are catalyzed by host proteases (signal peptidases), liberate sole protein component of the nucleocapsid, known as core protein (C), two envelope proteins (E1 and E2), and a strongly hydrophobic peptide, called p7, (the function of which remains unknown) [6, 123]. The remaining NS proteins are processed by viral proteases. A Zn^{2+}-dependent protease activity, that is associated with adjacent segments of the NS2 and NS3 proteins is responsible for the cleavage at the junction NS2/NS3 [78]. The protease cleaves the polyprotein only at this one site by an apparently autocatalytic mechanism [36]. Another serine-type viral protease activity is required for the cleavage at the COOH-terminus of NS3. This activity is associated with the NH_2-terminal domain of the NS3 protein [23, 74, 98] and is also responsible for the release of NS4A, NS4B, NS5A and NS5B. Proteolytic processing and release of the proteins does not take place consecutively along the ORF from 5′ to 3′. The apparently autocatalytic cleavage at the NS3/NS4A site as well as that at the NS5A/NS5B site occur rapidly and do not require any prior upstream events, however, the cleavages of the NS4A/NS4B and NS4B/NS5A junctions appear

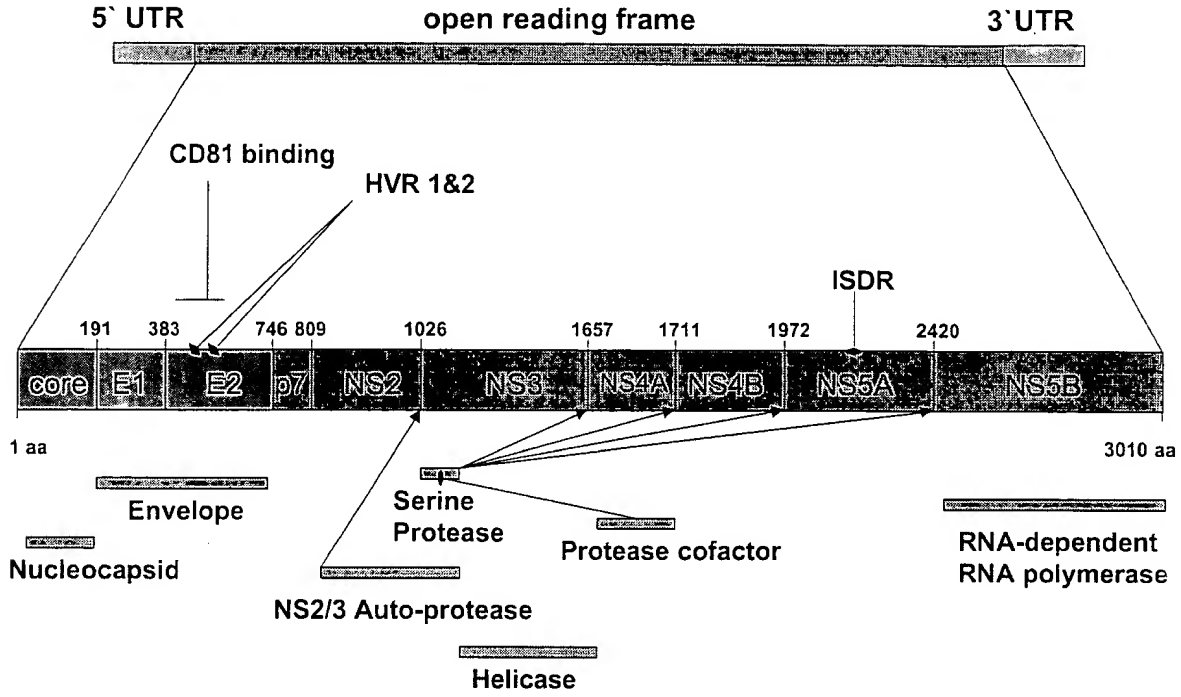

Fig. 1. Schematic representation of the structure of the HCV polyprotein. The upper narrow bar represents the genome of HCV, with the open reading frame (ORF) and untranslated regions (UTR) indicated. The lower wide bar represents the encoded HCV polyprotein, ca. 3010 aminoacids (aa) in size with the boundaries of the structural (light blue) and nonstructural (dark blue) proteins shown. The enzymatic activities associated with the non-structural proteins are indicated. The amino acid numbers on top of the lower bar are the locations of cleavage sites in the HCV polyprotein and the arrows point to the proteolytic cleavages performed by the autoprotease and the NS3 serine protease, respectively. Cleavages in the structural proteins are effected by host cell signalases. The two hypervariable regions of E2 (HVR 1 and 2), the putative binding site of HCV CD81 and the so called interferon determining region (ISDR) are also mapped in this schematic representation

somewhat delayed [5, 57]. Therefore, it is possible that the cleavage sites located 3′ of the NS3/NS4A site are processed both in *cis* and in *trans* [5, 57]. A simplified diagram of the genome organization of HCV with the cleavage sites in the mature HCV polyprotein indicated is shown in Fig. 1.

Features of HCV-encoded proteins and their interactions with intracellular protein phosphorylation

Core (C) protein

Core protein is a strongly basic (isoelectric point 12.05), evolutionarily highly conserved protein, which is the major protein constituent of the viral capsid and has RNA- and DNA-binding activity [88, 90]. At least two different forms of C protein have been described: p21c and p19c (corresponding to polypeptides 191-

and 173-amino acids long respectively). These two forms result from the cleavage at different sites in the HCV-polyprotein to form the COOH-terminus of C. Both proteins are localized in the cytoplasm and are associated with the endoplasmic reticulum (ER). A third form of the core protein, p16c (consisting of estimated 150 to 160 amino acids) has been described by some authors and was found only as a product of HCV genotype 1a. In contrast to the two other forms, p16 was localized in the nucleus [4, 60, 61].

Numerous research groups could demonstrate multiple biological effects in different cell lines that express C protein. For example, it was shown that C protein inhibited cisplatin-mediated apoptotic cell death in HeLa cells, and modified the expression of a broad range host cell and even other viral genes, both positively and negatively [89, 90, 104, 107]. Moreover, the expressed C protein is capable (alone and in cooperation with oncogenes like *H-ras* or *c-myc*) of inducing the transformation of numerous cell lines and of inhibiting apoptosis [90, 92]. The HCV core protein is a multifunctional protein that interacts with numerous cellular signal proteins like the lymphotoxin-ß receptor, tumor necrosis factor (TNF)-receptor 1, apolipoprotein AII, and the hnRNP and bZIP transcription factors [93 and references therein]. C protein also affects important cellular signal pathways that regulate the activities of nuclear factor kappa B (NF-kB), AP-1, SRE, mitogen-activated protein (MAP) and Raf-1 kinases, p53, signal transducer and activator of transcription (STAT) family proteins, and P21/waf1 [93 and references therein].

Many of these effects seem to be the result of indirect interactions between HCV C protein and intracellular protein kinases or their downstream effectors. For example, core protein expressed in NIH-3T3 cells binds to STAT3 and induces its tyrosine phosphorylation. STAT phosphorylation leads, in turn, to enhanced cell proliferation and even to anchorage-independent growth and tumorigenesis [121]. The exact molecular mechanism for the enhanced STAT-3 tyrosine phosphorylation remains unclear. It has been suggested that HCV core protein may reduce the accessibility of phosphorylated STAT3 to tyrosine phosphatases [121]. On the other hand, core protein, when expressed in HepG2 and NIH-3T3 cells, activates some protein kinases that possess tyrosine kinase activity such as extracellular signal-regulated kinase (ERK) and/or the p38 MAP kinase. Such a kinase activation could contribute to the enhanced phosphorylation of STAT-3 [25]. The last hypothesis will require further study, however, since the circulating C protein has also been reported to block ERK/MEK MAP kinase activation [120].

Another protein that directly interacts with HCV C protein is the 14-3-3 protein. The 14-3-3 proteins consist of a family of regulatory molecules that are involved in many different cellular processes, including mitogenesis, cell cycle control, and apoptosis [65]. 14-3-3 binding proteins (of which there are over 70) are very diverse and include kinases, phosphatases, receptors, structural proteins and transcription factors [27]. Most of the 14-3-3 ligands require a phosphorylation step before they can interact with their receptors. Both proteins (14-3-3 and C protein) form complexes in a phosphorylation-dependent manner (see below) and this leads to the activation of Raf-1 protein kinase and its downstream effector molecules (ERK's), which are key members of the Ras/Raf/MAP kinase signal

pathway [1]. One of the proposed mechanisms for Raf-1 activation proposes that there are short epitopes or structures in the HCV C protein that can mimic the domain(s) of activated Ras (Ras-GTP). These domains interact with Raf-1 and sustain its activated status [100]. On the other hand, since the C protein is capable of forming homodimers and higher homo-oligomers [66], it could not be ruled out that the bound 14-3-3 protein and/or Raf-1 promotes formation of active Raf–Raf homo-oligomers [115]. Recently, several studies have shown that regardless of the particular mechanism, activation of the Ras/Raf/MAP kinase pathway may contribute to the development and progression of HCC [43].

HCV C protein also interacts directly with intracellular protein kinases. The core protein has two recognition sites for cAMP-dependent protein kinase A (PKA): Ser-53 and Ser-116, and two sites for phospholipid/Ca2+-dependent protein kinase C (PKC): Ser-53 and Ser-99 [63]. Phosphorylation of these various residues has been demonstrated to regulate the biological activities of the core protein differently. Phosphorylation of HCV C protein on Ser-99 and Ser-116 appears to be essential for suppression of *Hepatitis B virus* (HBV) gene expression and replication in HuH-7 cells by HCV [105]. Furthermore, phosphorylation of Ser-116 regulates repression of the promoter of p21, the universal CDK inhibitor, (p21/waf1),44] by core protein and nuclear translocation of core protein [63]. However, phosphorylation at Ser-53 appears to be critical for the interaction between C and the 14-3-3 proteins mentioned above [1]. In this context it is noteworthy that PKC only recognized the phosphorylation sites on the truncated (p19) form of C [105]. One could hypothesize that the truncated C protein may have an altered structural configuration that would explain the differences in its biological activities. This hypothesis appears to be in agreement with the recent observation that HCV C protein that was expressed from HCC tumor tissue-derived 'sequences associated with interferon (IFN)-α, thereby inducing the double stranded RNA-activated protein kinase (PKR) and increasing its activity. However, core protein that was derived from non-tumor tissue from the same liver had no detectable effect on the activity of PKR and did not form any complexes with it. Although tumor and non-tumor derived core protein sequences differed by only a few amino acid residues, the mutations were sufficient to induce conformational changes that determined whether distinct C protein domains interacted with PKR or did not [22]. The last finding appears to be somewhat paradoxical (see below). There is ample evidence that PKR is a protein that blocks viral protein synthesis by phosphorylating the α-subunit of translation initiation factor (eIF2α) [85]. One could speculate that the lowered level of the HCV protein expression in tumor tissue that is a primary target for the immune response might support viral persistence. Further studies have to be conducted, however, to establish the biological relevance of the observation.

E1, E2 and E2-p7 glycoproteins

The glycoproteins E1 (30–35 kDa) and E2 (70 kDa) are consecutively released from the HCV polyprotein by host cell signal peptidases, but the cleavages

mediated by cellular signalases at E2/p7 and p7/NS2 are somewhat delayed and incomplete. This leads to the production of E2-p7-NS2 and later to products like E2-p7 and p7-NS2 in addition to mature E2, p7, and NS2 proteins (see below) [56]. No functional relevance has yet been attributed to this delayed and partially incomplete processing. The well documented export of p7 to the plasma membrane suggests that E2-p7, p7, or p7-NS2 may participate in the budding of assembled virus particles [17, 118]. Like many other viral envelope proteins, E1 and E2 are glycosylated at multiple Asn-residues. They form an E1-E2 heterodimer, which is held together by noncovalent interactions and probably by disulfide bridges as well [33]. Although the importance of the polymerisation has not been established, it has been suggested that the interaction between the proteins is necessary for their correct folding and thus, for the assembly of HCV virions [33]. Two regions displaying a very high variability, called hypervariable regions 1 and 2 (HVR1 and HVR2) are localized on the NH_2-terminus of the E2. These regions appear to be the only defined target for neutralizing antibodies [14, 101]. Several studies have demonstrated a correlation between the heterogenity of the HVR1 and the response to IFN-α treatment [14, 77].

The highly hydrophobic COOH-terminus of both glycoproteins is intercalated into the membranes of the ER and the ectodomains of both E1 and E2 are present in the lumen of the ER [70]. Another important function is associated with the COOH terminus of E2; there is a short amino acid stretch, localized between the residues 659 and 670 of the HCV polyprotein that is responsible for the inhibition of PKR by E2 [111]. This motif, called E2 phosphorylation homology domain (E2-PHD) is similar to the sequences surrounding the PKR autophosphorylation site(s) and to the eIF2α phosphorylation site (Ser 51 of its α-subunit) that is a target for PKR. Initial *in vitro* studies indicate that this E2 domain binds to PKR and, therefore, inhibits the autophosphorylation-stimulated kinase activity, by an intermolecular autophosphorylation reaction [112]. One could speculate that the effect of E2 on PKR-mediated phosphorylation might explain the differences in the response to IFN-α treatment [111]. However, recent studies have, demonstrated very low, if any, amino acid sequence variation of E2-PHD, so that the broad range of IFN-α treatment outcomes could not be explained by this mechanism [14]. An detailed analysis of the mode of inhibition of PKR by E2 might make the apparently paradoxical observation more understandable. Although eight amino acids of the sequence that contains the major autophosphorylation sites which contribute to full activation of PKR match exactly with the residues in the E2 protein, the inhibition of the kinase activity is not exclusively due to simple competition by E2-PHD. The fact that other regions of E2 and PKR might be involved in the protein–protein interaction and that E2 does not inhibit the autophosphorylation of all 14 sites (which are phosphorylated with the same efficacy *in vivo* [99]), make the mechanism of the inhibition more complex than a mere pseudosubstrate intermolecular interaction [112].

The most important feature of glycoprotein E2 is, however, that the ectodomain of the viral protein binds specifically to human CD81, a member of the tetraspanin superfamily of cell surface molecules characterized by four transmembrane

domains forming two extracellular loops [80, 94]. This binding plays not only a key role in HCV binding to and uptake by hepatocytes and other cells but also modulates essential cellular signal pathways. A recent report demonstrated that the cross linking of CD81 with E2 or with anti-CD81 antibodies blocks natural killer (NK) cell activation [21, 113]. This direct inhibition, accompanied by a "down-regulation" of cytokine production, was due to blocking of intracellular tyrosine phosphorylation in a way that is independent of the negative signalling pathway mediated by the well characterized src homology 2 domain bearing protein tyrosine phosphatase (SHP). The SHP signalling pathway is often involved in NK cell and other inhibitory receptor systems [16, 113]. In this context, it is surprising that the binding of E2 to CD81 on naive and antigen experienced human T cell subsets lowered their activation threshold for TCR-induced proliferation and cytokine production [116]. The binding of E2 to CD81 may also be responsible for the activation of the MAPK/ERK signal pathway in HepG2 cells [123]. These apparently contradictory effects of the interaction of CD81 with HCV-E2 might be explained by the fact that the CD81 is a component of multiple and distinct receptor complexes, that differ widely depending on the cell type examined [94].

p7-NS2 and NS2 proteins

Mature NS2 protein is a hydrophobic transmembrane protein with an apparent molecular mass of 23 kDa [23, 78]. Previous studies suggested that the COOH-terminus of the protein was in the lumen of the ER, whereas the NH_2 terminal region remained exposed in the cytoplasm [102]. Based on these observations and the fact that NS2 forms complexes with NS5A, it was postulated that the cytoplasmic part of NS2 served as an anchor for the proteins of the HCV replication complex in the ER. A recently presented model of the NS2 topology predicts, however, that both NH_2- and COOH-termini of the protein are localized in the ER lumen and that the protein contains four transmembrane domains separated by two short cytoplasmic stretches of 9 and 10 residues, respectively [118]. Thus, the anchor function of the protein remains to be established. The function of the p7-NS2 product that results from the delayed p7/NS2 site cleavage (see above) remains just as unclear: whereas a range of reports see p7 as the causative agent for the translocation of NS2 to the cellular membrane [17], recent work strongly suggested that the membrane association of NS2 is completely independent of p7 [118]. The major function of NS2 appears to be the cleavage of the HCV polyprotein at its own COOH-terminus by an autocatalytic zinc stimulated reaction that involves, along with NS2, the NH_2-terminal region of the NS3 protein [36]. Interestingly, the correct conformation is more important than the primary structure for successful cleavage of the NS2/NS3 site, suggesting that there is no true signal sequence [94]. To the current time there is no evidence that NS2 directly interferes with intracellular (or viral) protein phosphorylation events.

NS5A is a phosphoprotein occurring in two forms: p56 and p58 and it is widely accepted that p58 is a hyperphosphorylated form of p56 [110], and NS5A interacts with both NS2 and NS3 (see below). Production of functional NS2 by

autoproteolysis is necessary for the appearance of p58 [59]. Precisely how NS2 is involved in the generation of p58 has not yet been elucidated, but two mechanisms have been proposed. In the first scenario, NS2 induces conformational changes that lead to the exposure of additional phosphorylation sites for cellular protein kinases on NS5A, similar to the mechanism used by the so called "matchmakers" (see below) [87]. In the second scheme NS2 protects the phosphorylation sites on NS5A from attack by cellular phosphatases. However, it should be noted, that the involvement of other viral proteins in the formation of p58 form is controversial and may depend upon the particular HCV strain investigated (see below) [76 and references therein].

NS3 protein

NS3 is a slightly hydrophilic protein with a molecular weight of approximately 70 kDa. Multiple enzymatic activities are associated with this protein such as serine protease-, RNA-stimulated nucleoside triphosphatase (NTPase), and RNA helicase-activities [30]. The catalytic domain of the chymotrypsin-like NS3 protease, comprised of 180 amino acids, has been mapped to the NH_2-terminal region of the protein, whereas the NTPase and the helicase are located within the COOH-terminal 450 amino acids of NS3. The protein is released from the HCV polyprotein by autocatalytic cleavage at the NS3/NS4A site by the NS3 serine protease. As mentioned above, the same protease catalyses the cleavages at NS4A/NS4B, NS4B/NS5A and NS5A/NS5B sites; both in *cis* and *trans* [5, 57]. This proteolytic activity is precisely regulated by a cofactor: NS4A (see below) which is an integral part of the NS3 protease, as demonstrated previously by xray crystallography of the protease domain bound to the cofactor [49].

In vivo knock-out experiments with related flaviviruses and pestiviruses have shown that the NTPase and helicase activities associated with the COOH terminus of NS3 are essential for viral replication [34, 67]. The NTPase activity is activated by ds or ss RNAs and DNAs, particularly by poly(U) and poly(dU), which are inhibitors of the NS3 helicase activity [75, 108]. There are indications that this regulation, which has only been observed *in vitro* to the current time, might have an *in vivo* relevance since a direct binding and interaction between NS3 and the poly(U)-rich 3´NTR has recently been documented [3]. NS3 also interacts with NS5B very strongly; both proteins constitute major components of the replication complex and their helicase and RNA-dependent RNA polymerase (RdRp) activities respectively are essential for replication of the viral RNA [6, 42, 78].

In addition to its essential serine protease activity, the intact NH_2-terminus of NS3 fulfills an important regulatory function in the hyperphosphorylation of NS5A p56 and generation of its p58 form [76]. The presence of the authentic NH_2-terminus may be necessary for direct protein–protein interaction between NS3 and NS5A. Numerous laboratories have demonstrated the direct interaction between the NS3 and NS5 proteins of HCV, and the interactions between the comparable moieties of Dengue virus type 2 (DEN-2) and Japanese encephalitis virus (JEV) [18, 42, 45]. The mechanism by which NS3 promotes the hyperphosphorylation

of NS5A remains speculative. One possible mechanism is that there is a change in the conformation of NS5A as a result of interaction with NS3. This conformational change expose additional phosphorylation sites on NS5A and/or make the existing phosphorylation sites more accessible to a protein kinase. This action of NS3 is not without precedent. In our previous *in vitro* studies, NS3 was shown to strongly modulate the activity of PKC and to also affect the activity of PKA to a lesser extent. The extent of the modulation depends strongly on the concentrations of both NS3 and substrate; at higher concentrations of substrate and enzyme, hyperphosphorylation of both molecules occurred. There was a close correlation between the rate of binding of NS3 to substrates and the extent of their hyperphosphorylation [9, 11]. This mechanism of action is characteristic of a broad range of matchmakers (see above). "Matchmakers" are not phosphorylated themselves by protein kinases. They directly interact with their substrates, changing the conformation and biochemical properties of the substrates [87], as illustrated schematically in Fig. 2.

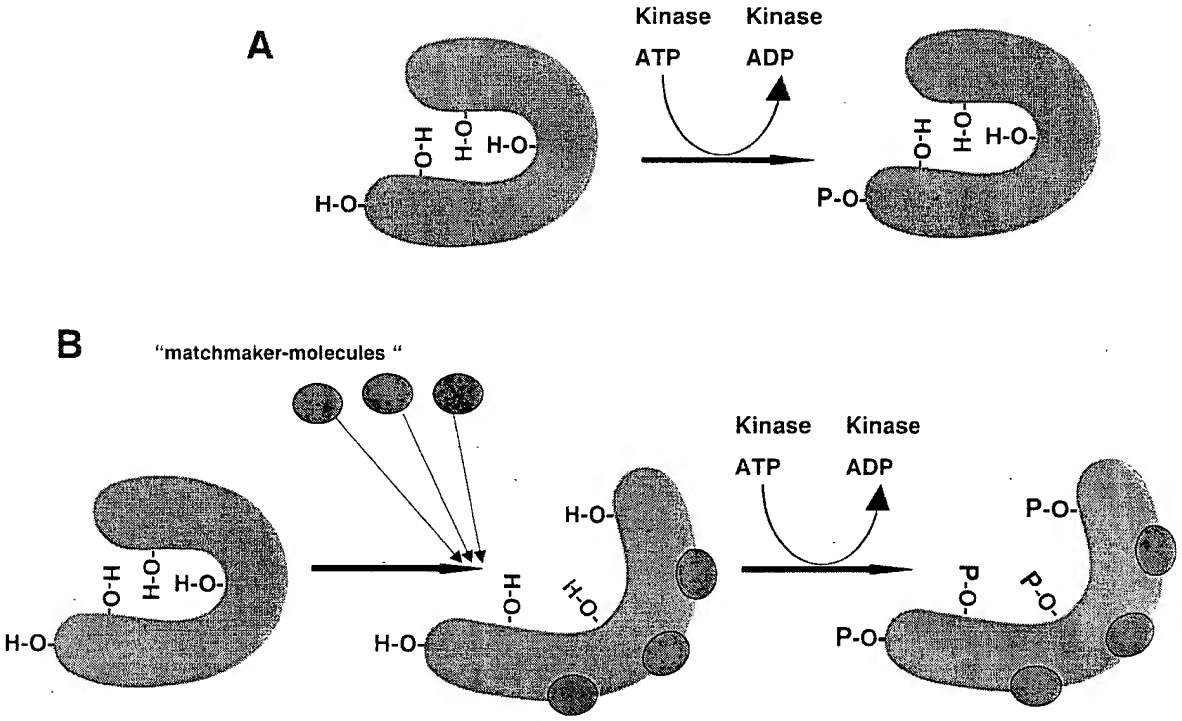

Fig. 2. Schematic presentation of the mechanism of action of matchmakers. **A** In the absence of matchmakers, the target molecule (red) is constrained in the "closed form" and only certain phosphorylation sites are available to kinases. **B** Matchmakers (presented here as blue ovals) bind to the (red) target molecule ("closed form") changing its conformation to the "open form". If the target molecule is a substrate for protein kinases, new phosphorylation sites made available or the existing sites are made more accessible on the "open form" in the presence of matchmakers (compare **A** with **B**). In some cases this leads to the hyperphosphorylation of the target molecule

However, the hyperphosphorylation of NS5A cannot be explained completely by the matchmaker mechanism mediated by NS3 alone. A wide range of viral proteins like NS4A, NS4B or NS5B, and currently unknown cellular proteins participate in the interaction between NS3 and NS5A. In the case of the NS viral proteins, it was well documented that they interact together with NS3 to generate the hyperphosphorylated NS5A p58 form. But the absolute requirement for these proteins for hyperphosphorylation is highly controversial and appears to depend upon the strain of HCV being used [59, 76].

In addition to its functions in the processing of the HCV polyprotein and in replication of the HCV RNA, NS3 has been demonstrated to affect intracellular signal transduction mediated by PKA and PKC. A short amino acid stretch localized between residues 1487 and 1500 of the HCV polyprotein resembles the autoregulatory pseudosubstrate domains of PKC and PKA and has similarities to substrate recognition sequences (consensus sequences) of both kinases [10, 12, 13]. PKC and the catalytic (C) subunit of PKA associate directly with NS3 through their catalytic sites and this interaction results in inhibition of kinase activity *in vitro* and *in vivo*. Furthermore, the NS3 binding seems to prevent the protein kinases from interacting with their intracellular target proteins *in vivo*. Two possible mechanisms for these effects can be envisaged. In the first case, direct interaction with NS3 could lead to immobilization and sequestering of the kinases in the cytosolic compartment. In the second case NS3, a large molecule, or fragments of NS3 could mask the binding sites on the kinases for their intracellular receptor(s) (in case of PKC the RACK's) [73] or mask signal sequences on the enzymes by steric hindrance, in either case preventing the translocation of the kinases to other compartments of the cell.

Kinetic analyses of inhibition of kinase activity as well as competition assays strongly suggested that domain(s) different from the arginine-rich sequence mentioned above are also involved in interactions between NS3 and PKC or PKA [10, 12]. It is noteworthy that the NS3-mediated inhibition of the kinase activity and the impairment of the free shuttling of PKC and PKA between the cell compartments are independent effects. A peptide corresponding residues 1487 to 1500 of the HCV polyprotein, or small fragments of NS3 containing this sequence, could inhibit the protein kinases *in vitro* and *in vivo*. However, in contrast to larger fragments of NS3 these small peptides did not affect the phorbol ester- or forskolin-induced redistribution of PKC or PKA. Moreover, they even co-translocated with the activated enzymes [10, 12].

NS4A and NS4B proteins

NS4A is a hydrophobic protein consisting of 54 amino acids. The protein has two important functions in the processing of the HCV polyprotein. First of all, NS4A acts as cofactor (activator) of the NS3-associated serine protease, and secondly, its NH_2-terminus anchors the replication complex to the ER by binding to NS3 [37, 42, 49]. Furthermore the protein–protein interaction of NS4A with NS3 promotes the interaction of NS3 with NS5B. On the other hand, NS4A may bind directly to

NS5B (with or without NS3) and enhance its polymerase activity, but the biological significance of that interaction remains to be elucidated [7, 42].

The NS4B protein has a molecular weight of approximately 30 kDa. There is little information available about the function of this protein in the processing of the viral RNA or the polyprotein. It is quite conceivable that (similar to the p7-NS2 and NS4A) the highly hydrophobic NS4B plays a role in the attachment of the replication complex to the ER [58]. The role of NS4A and NS4B in intracellular phosphorylation events or in protein phosphorylation-mediated signal transduction are only poorly characterized and remain controversial.

As mentioned above, NS4A and NS4B (expressed in *cis*) together with NS3 appear to be necessary for the hyperphosphorylation of the NS5A and generation of its p58 form in the BK strain of HCV [76]. However, in the HCV J strain NS4A alone (supplied in *trans*) was sufficient to generate the p58 form of NS5A [2, 110]. It is unclear, whether or not the differences in the induction mode of NS5A hyperphosphorylation correspond to a different degrees of phosphorylation of the nine conserved serine residues of the central region of NS5A [76]. It is also unclear at this time whether, the differing results are due to differences between the two NS5 proteins from different HCVstrains or whether they are due to differences in the experimental conditions used.

NS5A and NS5B proteins

NS5A is a hydrophilic phosphoprotein which is primarily phosphorylated on serine residues, but also to a lesser extent on threonine residues. As mentioned above it occurs in two forms: one with an apparent molecular mass of 56 kDa (NS5Ap56) and a second, with an apparent molecular mass of 58 kDa (NS5Ap58) which is a hyperphosphorylated form of NS5Ap56 [110]. The functions of NS5A in the processing of the viral RNA or HCV polyprotein are still to be elucidated. Nevertheless, there is accumulating evidence that this protein interferes with intracellular signal transduction. Direct interactions of NS5A with signalling proteins like p53, cyclin-dependent kinase (Cdk)1/2-cyclin complex, or the Snf2-related CREB-binding protein activator protein (SRCAP), an ATPase that enhances the ability of the CREB-binding protein to function as a coactivator for a number of transcription factors, have been reported [97 and references therein]. NS5A localized in the ER activates the release of calcium from ER stores, thereby inducing oxidative stress with concomitant activation of STAT-3 and NF-kB [31]. Furthermore, there are indications that the NS5A protein may interrupt signal cascades more directly. In HeLa cells infected with recombinant vaccinia virus expressing NS5A, The viral protein bound directly and specifically to the growth factor receptor-bound protein 2 (Grb2) adaptor protein. This binding was mediated by the proline-rich motifs of NS5A that strongly resemble the binding sites for the src homology (SH) 3 domain. This interaction caused a down regulation of the basal and growth factor-induced-phosphorylation mediated by the extracellular signal-regulated kinases 1 and 2 (ERK1/2). It is therefore possible that NS5A may disrupt the ERK-mediated mitogenic signalling [109].

As mentioned above, NS5A serves as substrate for cellular kinase(s) itself. The basal form (NS5Ap56) is phosphorylated on multiple serine and to a lesser estent on threonine residues located downstream of tamino acid 2350 (in the HCV-J strain) [96, 110]. Other phosphorylation events at residues in the central region of NS5A (Ser-2197, Ser-2201, and Ser-2204) are required for hyperphosphorylation and the generation of the p58 form of the protein [110]. At least two additional major phosphorylation sites have been identified; the first is the highly conserved serine 2194. And the second is serine 2321, that appears to be specific for genotype 1a [47, 96]. Although the biological role of phosphorylation and hyperphosphorylation of NS5A remains to be clarified, it is a common event for all HCV strains tested and for other members of the family *Flaviviridae*, implying its importance in the life cycle of these viruses. Studies performed with HeLa cells coinfected with recombinant vaccinia virus encoding Dengue virus NS3 and NS5 proteins give some further insight in these processes. The two dengue proteins formed a complex which associated with the ER. Subsequently NS5 was hyperphosphorylated by a cellular kinase(s), dissociated from NS3, and migrated to the nucleus. The function of the nuclear pool of dengue NS5A is unknown. One could hypothesize that NS5A may regulate the expression of cellular genes in response to viral infection. It is noteworthy that no other flavivirus protein seemed to be necessary for the interaction [45]. These flavivirus results show significant parallels to the intracellular processing of HCV NS5A. Certain NH_2- and/or COOH-terminal cleavages of NS5A "unmask" a functionally active nuclear localization signal (NLS) which aids in translocation of the truncated protein to the nucleus [40, 103]. Moreover, domains of NS5A appear to participate in transcriptional activation and/or repression of genes [46].

The kinase(s) that is/are responsible for the phosphorylation of NS5A has not been identified. The enzyme appears to be evolutionarily well conserved since it occurs in yeast as well as insect and mammalian cells [47]. A number of experiments suggest that a member of the so called CMGC group of serine threonine kinases, the casein kinase II (CKII), or the catalytic subunit of PKA could be responsible [41, 50].

The best characterized function of NS5A is its ability to interact with PKR and thereby to block the phosphorylation of eIF-2α (see above). The mechanism of action of NS5A appears to be slightly different from that postulated above for E2. NS5A possesses a short amino acid stretch called IFN-α sensivity-determining region (ISDR), localized between amino acids 2209 and 2248 of the HCV polyprotein [24], which interacts with a well defined PKR dimerisation domain localized between amino acids 244 and 296 of the kinase [29]. But this interaction NS5A prevents the dimerization of PKR that is necessary for the catalytic activity of the protein kinase, and therefore blocks the PKR-mediated phosphorylation of eIF-2α [29]. From this model, it was hypothesized that HCV strains with mutated ISDR sequences such that they could not bind to PKR would remain susceptibe to the antiviral effect of IFN-α [24, 29, 52]. However, no correlation appears to be between the sequence of the ISDR and IFN-α sensitivity

[48, 81]. This discrepancy may be explained by the fact that other HCV proteins and/or other domains of NS5A also affect PKR-mediated phosphorylation (see above and [109]).

The inhibition of PKR by NS5A (and E2) appears to play an important role in the pathogenesis of HCV infection. This protein kinase, in addition to its antiviral activity, activates NF-kB and IFN-regulatory factor 1 (IRF-1); PKR has also been characterized as a tumor suppressor and as a regulator of gene expression in specific apoptotic programs [29, 51, 69, 85]. Therefore, disruption of the PKR-mediated signal transduction may contribute to the oncogenicity of HCV [29].

NS5B is a 68 kDa protein possessing conserved motifs characteristic of RdRp and this replicase has been extensively characterized in numerous laboratories [7, 38, 53, 79, 106]. The relative low turnover rate of NS5B RdRp in comparison with the poliovirus 3D polymerase, for example, may suggest that the enzyme requires viral and/or cellular cofactors. Indeed, NS5B has been demonstrated to directly interact with NS3, NS4A, and other components of the replication complex [2, 38, 42, 79, 106], and that these interactions regulate RdRp activity [79]. A few reports have suggested that NS5B contains terminal nucleotidyl transferase activity but this result need to be confirmed. Full-length NS5B is localized in the cytosolic perinuclear space, associated with ER membranes [37], but the COOH-truncated form of the protein is translocated to the nucleus. The biological significance of the nuclear translocation is unclear [119]. In contrast to NS5A there is only limited evidence for the intracellular phosphorylation of NS5B. Baculovirus-expressed NS5B has been shown to be phosphorylated in insect cells [30], but the phosphorylation could not be reproduced in mammalian cells. The effect of phosphorylation on the subcellular localization and on the RdRp activity of NS5B is unclear.

Conclusions

Although 170 million people are chronically infected with HCV, options for therapy are still limited. This is due primarily to the absence of a suitable cell culture system or a convenient animal model and the fact that the virus is extremely variable; due to the high mutation rate abundant quasispecies that express proteins differing in their biochemical and biological properties can be found in patients. Consequently, all available information on interaction of the different HCV proteins with the cell has been obtained from ectopic expression of viral antigens in cell culture. Whether or not these experiments acurately depict the course and pathophysiology of natural HCV-infection remains to be verified. This question also has to be raised in the broad range of interactions between the intracellular phosphorylation of proteins and the viral antigens described above. The possibility to infect the cells with native HCV appears, in this context, very promising [26]. This cell culture system allows not only to confirm the pathophysiological function of the viral antigens, but also to develope appropriate strategies for intervention.

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